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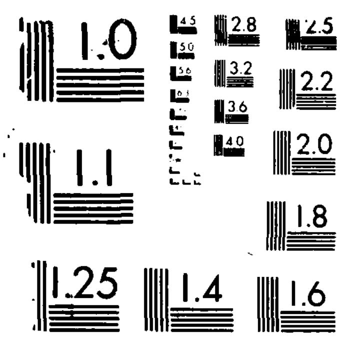

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EFFECTS OF ORGANOPHOSPHATE ESTERS ON NEUROPEPTIDE SYSTEMS

ANNUAL REPORT

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Organophosphate esters were studied in terms of their ability to affect neuropeptides in the rat central nervous system. The in vivo biosynthesis of enkephalin peptides in the basal ganglia and of vasopressin and oxytocin in the hypothalamus were studied and the effect of subcutaneous administration of diisopropylfluorophosphate (DFP) in the biosynthesis of these neuropeptides was assessed. It was found that DFP inhibited the biosynthesis of vasopressin and oxytocin. These results suggest that organophosphates exert their effects not

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20. Abstract (continued)

only on cholinergic systems but also on neuropeptide systems important in endocrine and cardiovascular function.

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SUMMARY

The purpose of the work being carried out under this contract is to explore the hypothesis that the toxic effects of organophosphate esters in humans may be contributed to by actions of these compounds exclusive of those due to their inhibition of cholinesterase enzyme activity, specifically by actions on peptide-secreting neurons in the central nervous system. The rationale for pursuing such an hypothesis was two-fold: (1) in the last 10 years it has become evident that peptide-secreting neurons comprise a major and hitherto unknown cellular element in the vertebrate nervous system, and that their distribution suggests major regulatory roles for neuropeptides in physiological processes such as cardiovascular, respiratory, autonomic and endocrine function, major targets for organophosphate toxicity, and (2) the biochemical pathways for the synthesis and degradation of neuropeptides involve hydrolytic enzyme reactions which may be mechanistically similar to those catalyzed by cholinesterases, and that hence organophosphates could also act to disturb these neuropeptide systems.

If such targets of organophosphates can be defined biochemically, as intended in the present work, then subsequent physiological studies can be carried out to determine to what extent the effects on neuropeptide systems contribute to the toxicity exerted by organophosphates. If this is significant, then: (a) a new target for organophosphate toxicity has been defined and (b) a possibility exists for novel routes to reversal of the toxic effects, via neuropeptide agonists or antagonists. The overall implication is new routes to benefit the soldier in the field who may be subjected to organophosphates.

The approach taken to investigate this hypothesis was to establish systems in which to carry out direct studies of the biosynthesis of certain neuropeptides *in vivo*, in the rat, and of neuropeptide processing and degradation *in vitro*, and to assess the effect of introducing organophosphates into these systems. The methods used for the biosynthetic studies involved infusion of radiolabeled amino acids stereotaxically into the site of the cell bodies of origin of two important neuropeptide projection systems: the caudate nucleus to globus pallidus enkaphalin system, involved in the extrapyramidal regulation of motor activity, and the major efferent projections of the paraventricular nucleus of vasopressin and oxytocin secreting neurons of the hypothalamus: (a) to the posterior pituitary gland for regulation of plasma volume (b) to the nucleus tractus solitarius in the brainstem for respiratory and cardiovascular regulation and (c) to the preganglionic cell column of the spinal column, for regulation of peripheral automic function. Following labeling, the respective peptides enkephalins, vasopressin and oxytocin are purified from the terminal fields and the rate of biosynthesis determined for control and organophosphate. During the period covered by this report, we have established a working organophosphate laboratory after some initial delay in reconstruction, and have completed baseline studies for successfully being able to carry out the intended biosynthetic studies. In conclusion we are now in a position to assess organophosphate effects on important neuropeptide systems using a dynamic and sophisticated biochemical approach.

c. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

d. In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

I. Statement of the Problem

To investigate the hypothesis that some of the toxic effects of organophosphates on the nervous system may be exerted on nerve cells which utilize neuropeptides as neurotransmitters or neuromodulators to regulate key physiological processes, as well as on neurons which utilize acetylcholine.

II. Background

Most organophosphate esters (OPE) are known to inactivate the enzyme aceetylcholinesterase (AChE) by alkylation, and to give rise to a variety of toxic effects on the nervous system in vivo, the most severe of which impair the function of cardiovascular and respiratory control centers in the brainstem.[1,2] While it is believed that both the lethal effects and the debilitation observed in survivors of OPE poisoning are due to an excess of the neurotransmitter acetylcholine (ACh) in nervous tissue, it is by no means true that it has been unequivocally established that the morbidity resulting from exposure to OPE is due exclusively to effects on cholinergic systems. Results emerging from research in neurobiology over the last 10 years provide a basis for considering a possible additional locus of OPE toxic effects: peptidergic neuronal systems. This research has defined the existence of a large number of previously unknown neuron systems which utilize peptides as neurotransmitters or neuromodulators (neuropeptides).[3] These neuropeptides undergo two modes of metabolic transformation which involve hydrolytic enzymatic reactions which may be mechanistically similar to that catalyzed by AChE: biosynthesis, in which the biologically active neuropeptides are liberated from higher molecular weight precursors by proteolytic cleavage[4], and inactivation, in which the mature neuropeptides are hydrolyzed by peptidases[5]. More importantly, neuropeptides are distributed in the nervous system so as to influence all of its integrative activities.[3] With regard to OPE toxic effects, a number of different neuropeptide systems appear to contribute to the regulation of cardiovascular and respiratory function; e.g. the projection of oxytocin and vasopressin secreting neurons from the paraventricular nucleus of the hypothalamus to the nucleus tractus solitarius and the dorsal motor nucleus of the vagus;[6] to endocrine regulation, e.g. hypothalamic projections of vasopressin and oxytocin neurons to the posterior pituitary gland[7], and oxytocin and enkephalin-secreting neurons to the median eminence[8] ; to the regulation of movement, e.g. the projection of enkephalin-secreting neurons from the caudate nucleus to the globus pallidus[9], and to the regulation of peripheral autonomic function, e.g. the projection of oxytocin and vasopressin-secreting neurons from the paraventricular nucleus of the hypothalamus to the preganglionic cell column in the thoracic and lumbar segments of the spinal cord.[10] If OPE acted on either the biosynthetic or degradative enzymes, or both, for the neuropeptides in these projection systems, it could result in a dis-integration of the regulatory actions of these systems which could contribute to the toxic actions of these agents. At the present time, there is virtually nothing known about the impact of OPE on neuropeptides. In light of the above discussion, direct biochemical studies of the effects of OPE on neuropeptide biosynthesis and degradation would constitute a foundation for understanding alterations in the physiological processes regulated by neuropeptides caused by OPE. It is to this end that the studies comprising this contract have been designed.

III. Approach to the Problem

The problem was approached by first attempting to establish *in vivo* systems for studying the biosynthesis of neuropeptides in pathways related to cardiovascular, respiratory, endocrine, autonomic and locomotion regulation, specifically: (1) vasopressin and oxytocin in the hypothalamic paraventricular nucleus to posterior pituitary, brainstem and spinal cord projection systems; (2) the biosynthesis of oxytocin and enkephalin in the paraventricular nucleus to median eminence projection system and (3) the biosynthesis of enkephalins in the caudate nucleus to globus pallidus projection system in the corpus striatum. This would then comprise a baseline for subsequent studies in which OPE were introduced into the animals prior to biosynthesis studies.

In attempting to estblish these *in vivo* biosynthesis used in biosynthetic studies on other neuropeptide systems, such as Luteinizing Hormone Releasing Hormone[11], proopiomelano-cortin[12] and others. This approach involves stereotaxic introduction of cannulae to overlie the site of cell bodies of origin of a given neuronal projection system, infusion of radiolabeled amino acids to this site through tubing connected to a subcutaneously implanted osmotic minipump, harvesting the tissue containing the terminal field of the projection system under study after sufficient time for synthesis and transport of the neuropeptides to that site and purification to homogeneity of the labeled (newly synthesized) neuropeptides by sequential High Performance liquid chromatography (HPLC) coupled with chemical modification.[13]

The establishment of these baseline *in vivo* systems was the immediate goal of the period covered by this report.

IV. RESULTS AND DISCUSSION

1. Vasopressin and oxytocin biosynthesis and transport to posterior pituitary, brainstem and spinal cord.

We had previously demonstrated the biosynthesis and transport of vasopressin and oxytocin to the posterior pituitary and brainstem. However, it was necessary to develop a system for studying the synthesis and transport of these neuropeptides along the extremely long monosynaptic projections to the spinal cord, and to be able to measure the labeling in all three sites in a single experiment. We experimented with the duration of isotope infusion and the post infusion transport time in order to be assured of observing the synthesis of both peptides. Using a 2 hour pulse and 10 and 48 hour chase times, and continuous infusion for 18 hours, we demonstrated a differential rate of labeling of oxytocin and vasopressin both the thoracic and lumbar projections of these neurons. Oxytocin synthesis and transport to thoracic and lumbar spinal cord could be detected using a 2 hour pulse of ^{35}S-cysteine and a 10 and 48 hr, respectively, chase time, while no vasopressin synthesis could be detected under either of these two conditions. When a continuous isotope infusion of 16 hours was carried out, labeled vasopressin could be detected in both thoracic and lumbar cord. It was also determined that oxytocin is transported by the rapid system of axonal transport (>8mm/hr) in this extremely long projection system. In addition, the neurophysin proteins were also found to be synthesized and transported down the spinal cord along with their associated nonapeptides. In these experiments in which successful labeling of the spinal cord projections was achieved, we found adequate labeling of the posterior pituitary and brainstem projections of oxytocin and vasopressin secreting neurons arising in the paraventricular nucleus of the hypothalamus. Thus, we have successfully worked out a system for direct study of the synthesis and transport of neuropeptides believed to be involved in the regulation of endocrine, cardiovascular and respiratory function. A paper describing these results is in press in Neuroscience. A copy of the paper is included in the Appendix.

2. Enkephalin and oxytocin biosynthesis processing and transport in the hypothalamic paraventricular nucleus-median eminence and posterior pituitary projection systems.

As indicated earlier in this report, the co-localization of 2 different neuropeptides-oxytocin and enkephalin-individual neurons in the paraventricular nucleus of the hypothalamus offers the opportunity of studying the effects of organophosphate toxins on the biosynthesis of 2 different peptides, in the same cell, in the same experiment. It was first necessary to establish the ability to measure the biosynthesis of these peptides in vivo. We chose the female rat because it could be expected that lactation would provide a stimulus for oxytocin biosynthesis, and possibly enkephalin biosynthesis, so that the effect of OPE could be studied on a basal and a stimulated system. Animals were cannulated over the paraventricular nucleus and ^{35}S-methionine and ^{35}S-cysteine were administered via indwelling cannulae. We found that a series of 3 successive gradient elution steps on HPLC were sufficient to achieve purification of several labeled enkephalin peptides: methionine^5-enkephalin (MENK), Met^5 -Arg^6 -Gly^7 -Leu^8-enkephalin (MERRGL) and Met^{5}Arg^6 -Phe^7-enkephalin (MERF), as well as labeled oxytocin and vasopressin. It was found that enkephalin was transported to the median eminence preferentially to the posterior pituitary, that the stimulus of lactation resulted in the same enhancement of enkephalin as oxytocin biosynthesis, and that the ratio of MENK:MERGL:MERF did not change during stimulation of enkephalin biosynthesis by lactation. These results provide direct biochemical evidence that enkephalin and oxytocin are co-regulated in PVN neurons, that the median eminence is the major terminal field to be analyzed for PVN enkephalin neurons, and that processing of proenkephalin is regulated during stimulation. The effect of OPE administration can now be examined on all of these behaviors of the system.

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V. Conclusions

Successful baseline studies have been carried out for subsequent studies on the effects of OPE on the biosynthesis of neuropeptides in neuronal pathways implicated in the regulation of endocrine, respiratory and cardiovascular function. This involved the successful purification to homogeneity of radiolabeled peptides following *in vivo* infusion of ^{35}S-cysteine and for ^{35}S- methionine into sites of cell bodies of origin of the following projection systems in rat brain: (1) the hypothalamic paraventricular nucleus projections of oxytocin and vasopressin neurons to the posterior pituitary gland, the nucleus tractus solitarius and dorsal motor nucleus of the vagus, the autonomic preganglionic cell column of the spinal cord; (2) the projection of oxytocin and enkephalin neurons from the hypothalamic paraventricular nucleus to the median eminence in control and stimulated states of activity of these paraventricular neurons.

VII. Recommendations

Based on the successful completion of these baseline studies, the impact of organophosphate agents of military significance on these neuropeptide systems which contribute to the regulation of physiological systems most affected by organophosphate toxicity should now be assessed.

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